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Rapid Sample Preparation for Determination of Iron in Tissue by Closed-Vessel Digestion and Microwave Energy

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We developed a rapid acid-digestion method for preparing tissue samples for iron determination. Specimens were digested in nitric acid and hydrogen peroxide under high temperature and pressure in closed Teflon vessels, with microwave energy. Analysis for iron in 25- to 250-mg portions of digested bovine liver powder (National Bureau of Standards Certified Reference Material no. 1577a) showed excellent linearity ([predicted] = 1.007[actual] − 0.166 μg per sample) and analytical recovery (98%). Precision (CV) was 5.4% when iron content was 10 μg per sample. Assaying split samples of mouse tissues, we found a close correlation between iron concentrations obtained with closed vs open vessels ([closed] = 0.878[open] + 68 μg/g, $r = 0.994$, range 400–4600 μg/g dry weight). In contrast to time-consuming conventional procedures for tissue dissolution, closed-vessel digestion with microwave energy dramatically shortens time for tissue preparation, minimizes use of caustic acid, reduces risk of sample loss or contamination, and yields accurate and reproducible results.

Additional Keyphrases: *tissue analysis · hemochromatosis · anemia*

Recent evidence (*1*) that U.S. males are more commonly afflicted by homozygous hereditary hemochromatosis than by iron-deficiency anemia underscores the need to identify and reliably quantify tissue iron excess in iron-loading syndromes. Although determination of iron concentration in biopsies of liver remains the accepted standard for assessing iron stores in hemochromatosis, procedures currently available for processing tissue before iron assay suffer significant methodological drawbacks.

Conventional tissue-ashing techniques, which rely on prolonged exposure to heat and caustic acids to achieve complete tissue decomposition in open vessels, are time-consuming, prone to sample loss and contamination, and likely to release hazardous oxidative fumes (*2–4*). For these reasons, and because gaining reliable results requires rigid adherence to proper technique, such open-vessel digestion procedures are not readily adapted to routine clinical laboratory testing.

Applying microwave energy to samples in closed Teflon PFA (perfluoroalkoyl) vessels in the presence of acid creates high pressures and temperatures, rapidly promoting complete sample dissolution (*5*). Although detailed theoretical considerations suggest that rapid digestion of biological materials is possible (*6*), information about the practical application of this technique is lacking.

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Here we describe a closed-vessel, microwave digestion method for preparing tissue samples before iron determination. The method is rapid and safe; requires no special air-purification equipment, reagent purification, or personnel training; and is suitable for routine clinical laboratory testing.

Materials and Methods

Materials

Apparatus. The digestion system consisted of a Model MDS-81D microwave oven (CEM Corp., Indian Trail, NC) equipped with a 600-W power source adjustable from 0 to 100% in 1% increments and programmable in three stages, a rotating turntable, a Teflon-lined cavity and high-volume exhaust blower, twelve 60-mL Teflon PFA digestion vessels with caps, high-pressure relief valves, collection vessel and tubing, and powered fixed-torque capping station (all from CEM). Iron content of samples was determined with a Model 603 atomic absorption spectrophotometer (Perkin Elmer Corp., Norwalk, CT), with use of a hollow-cathode light source at 248.3 nm, a slit setting of 0.2 nm, and a lean air–acetylene flame.

Reagents. Concentrated "analyzed"-grade nitric acid (Baker Chemical Co., Phillipsburg, NJ) and 30% "analytical" grade hydrogen peroxide (Mallinckrodt, Inc., St. Louis, MO) were used in the digestion procedure. Reagents for cleaning labware were reagent grade. We use water of 18 MΩ resistance, produced by the four-bowl Milli-Q water treatment system (Millipore Corp., Bedford, MA).

Standards. National Bureau of Standards bovine liver powder, Certified Reference Material (NBS CRM) 1577a (National Bureau of Standards, U.S. Department of Commerce, Gaithersburg, MD), was used as a tissue standard for iron determination. Iron standards for atomic absorption analysis were made up from a 1 mg/L iron standard (Spex Industries Inc., Edison, NJ) in dilute (20 mL/L) HNO_3.

Labware. Dilutions of the 1 mL/L iron standard were prepared and stored in acid-washed Pyrex (Corning Glass Works, Corning, NY) Class A volumetric flasks. No other labware except for digestion vessels (above) was used. To prepare glassware for use, we soaked it in 6 mol/L HCl for 30 min, rinsed in water, soaked in 8 mol/L HNO_3 for 48 h, and again rinsed with water. We cleaned the digestion vessels as follows: to each vessel add 10 mL of 10 mol/L NaOH, swirl, and decant; wipe the vessel dry, then rinse with water; add 2 mL of concentrated HCl, cap, and place the vessel in a microwave oven to digest any residual impurities for 15 min at 25% power; rinse, then repeat the digestion procedure but use 2 mL of concentrated HNO_3. Finally, rinse the vessel three times with water and place it in an iron-free container to dry (we use plastic sweater boxes).

Laboratory conditions. The digestion apparatus was bench-mounted and vented via a flexible duct to a standard chemical fume hood. All vessels were sealed and unsealed in the fume hood. The air supply to the laboratory had no special filtration or purification treatment.

Procedures

Closed-vessel digestion with microwave energy. Perform all procedures with digestion vessels. Include one empty vessel (the reagent blank). Dry each vessel while empty in the microwave oven at 60% power for 15 min, then weigh immediately (empty-vessel weight). Add the wet tissue sample and weigh (sample wet weight). Place the relief valve on each vessel without capping and dry in three stages: at 15% power for 35 min, then 35% for 20 min; at 40% for 20 min, then 50% for 10 min; and finally at 60% for 10 min. Weigh, and subtract the weight from heated vessel weight to obtain dry tissue weight. Add 1 mL of 16 mol/L HNO_3 and 1 mL of concentrated (30%) H_2O_2 solution to each vessel, position the relief valve and cap, tighten the cap by using the capping station, and position the capped vessels on the turntable with the vent tubes attached to the collection vessel. Place the turntable in the microwave oven and digest samples for 15 min at 25% power. Cool the vessels for 30 min, then uncap and add 25–50 mL of water, taking care to rinse cap, pressure-relief valve, and vessel sides. Record weight (full-vessel weight), and calculate total sample volume by subtracting empty-vessel weight from full-vessel weight. Determine the iron concentration in the diluted digestate by atomic absorption spectrophotometry, multiply by total sample volume, and divide by the sample's dry weight to obtain the iron concentration of the sample.

Open-vessel digestion by wet ashing. Split samples of approximately equal wet weight were obtained from organs of mice known to show spontaneous tissue iron overload and prepared for analysis by open-vessel digestion as previously reported (7). Before assay, dry Pyrex borosilicate glass 30-mL beakers for 6 to 9 h at 105 °C and weigh. Add the samples and record the wet weight. Dry the samples at 105 °C for 24–72 h and record the dry weight. In a chemical fume hood add 2 to 6 mL of concentrated HNO_3 to each beaker, cover with a watch glass for 16–24 h or until sample appears dissolved, then add 2 mL of 30% hydrogen peroxide, place the mixture on a hot plate and boil dry. Repeat the acid–peroxide steps if solids remain. Finally, add 10 to 50 mL of water and determine the iron content of digestate as described above.

Analytical-recovery studies. We determined, by both nonradioactive and radioactive methods, how much iron was accounted for after closed-vessel microwave digestion. Recovery of nonradioactive iron was determined by adding known volumes of the 1 mg/L atomic absorption iron standard to weighed dry samples of CRM 1577a, measuring the total iron content after sample digestion, subtracting the iron content attributable to sample alone, and calculating the percentage recovery as (amount found of added/amount added) × 100. We also determined recovery of ^{59}Fe in samples of liver obtained from mice after repeated intraperitoneal injection with a total of approximately 20 μCi of $^{59}FeSO_4$ sulfate (New England Nuclear, Boston, MA). We measured the radioactivity of each sample in a gamma counter (Packard Instruments, Downers Grove, IL) before and after tissue digestion and compared the results after correction for geometry.

Results

Rapid digestion of tissue samples weighing less than 250 mg by the closed-vessel method yielded a slightly yellow digestate, free of detectable lipid. In 89 samples of CRM 1577a bovine liver powder weighing 25 to 250 mg and processed in 13 mixed-weight batches of four to 12 samples, we determined the mean iron concentration to be 203.3 (SD 6.5) μg/g; the NBS-certified iron concentration was 194 (range 174–214) μg/g. Agreement between certified and measured values was maintained over a wide range of sample size (Figure 1).

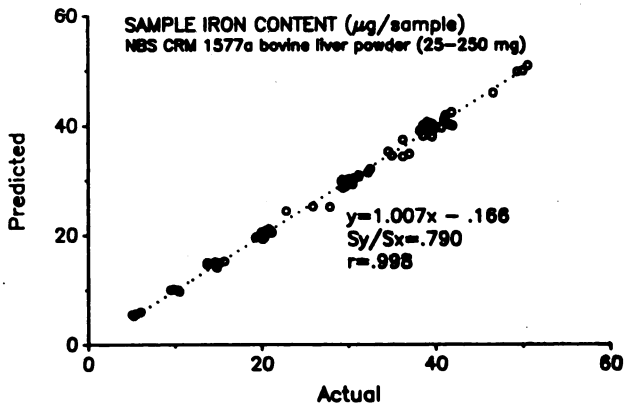

Fig. 1. Correlation between iron content (μg per sample) of 89 samples of bovine liver powder (NBS CRM 1577a) prepared by closed-vessel digestion with microwave power and that predicted by multiplying sample weight by mean sample iron concentration (203.3 μg/g, compared with NBS-certified value 194 ± 20 μg/g) over a wide range of sample size (25–250 mg)

The average CV was 3.2% for all 89 samples, and ranged from 1.1% for the 200- to 250-mg (40–50 μg of total iron) samples to 5.4% for 25- to 50-mg (5–10 μg of iron) samples. Average within-run precision (CV) in the NBS samples weighing 100 to 250 mg was 1.8% (mean measured iron concentration 202.6 μg/g). The precision (CV) of repeated measurements of dilute iron standards (2 μg/mL) by the atomic absorption spectrophotometer during sample processing was routinely less than 1%.

To compare results of the current method with those of previous dissolution techniques, we examined the concentration of tissue iron in 21 split samples from liver, spleen, and kidney obtained from mice with inherited iron overload and from normal controls. Samples digested in the closed-vessel digestion system yielded mean values slightly lower [by linear regression: closed = 0.878 (SD .023)open + 68 (SD 45) μg/g, $S_{y/x}$ = 150 μg/g, range 400–4600 μg/g] than those digested by ashing in open vessels. However, this difference was not statistically significant (P = 0.08 by Student's paired t-test).

Recovery of added nonradioactive iron was 98.8% (SD 2%) (nine samples) and of radioactive iron was 97.5% (SD 4%) (n = 10 samples).

Discussion

Digestion in closed vessels with microwave energy permits rapid, accurate, and precise determination of iron concentrations in tissue. The procedure outlined requires small volumes of inexpensive, commercially available reagents that need not be distilled before use and pose little hazard for handling or disposal. Neither ambient air purification nor extensive training in techniques of contamination control is necessary for reliable assays.

Limiting the sources of contamination contributes to both accuracy and precision gained by the present procedure. Contamination from labware is minimized by performing all steps in a single Teflon vessel, which is later acid-

washed, with use of microwave energy; contamination from reagents is minimized by restricting the total predilution reagent volume to 2 mL per sample; and airborne (positive) or sample-loss (negative) contamination is avoided by using closed vessels.

Drying the sample is the most critical and time-consuming step in the digestion procedure. Although the microwave oven provides faster drying than lyophilizing or heating in a conventional oven, the wet tissue samples must be heated slowly by stepwise increase in microwave power to prevent violent volatilization and potential loss of sample. Using the present protocol, we usually but not invariably obtained stable sample weight after the second power cycle; the lower the initial sample wet weight, the sooner a stable dry weight was achieved.

Needle biopsies of liver yield 2 to 20 mg of dry tissue (*8, 9*). Iron content in biopsies from patients with iron overload often exceeds 10 μg per sample. The precision and sensitivity of the closed-vessel digestion method for iron content in the range of 5 to 10 μg suggest that the method is well suited for measurement of hepatic iron in needle-biopsy specimens. Potential drawbacks of the method include the initial cost of the equipment (approximately $7500) and the likelihood that the digestion protocol will have to be adjusted for each laboratory as well as for each new element assayed, including hepatic copper in Wilson's disease and osseous aluminum in renal osteomalacia.

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